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Ribonuclease of Bovine Milk: Serological Relationship to Pancreatic Ribonuclease

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Ribonuclease isolated from bovine milk has been shown to be serologically identical to pancreatic ribonuclease. The presence of ribonuclease in milk was demonstrated directly by a gel-diffusion method. The serological method employed did not detect ribonuclease in bovine serum. Two distinct contaminants were detected in a commercial sample of pancreatic ribonuclease.

On the basis of chromatographic experiments, Martin and Porter (1) proposed that bovine pancreas contains two proteins with ribonuclease (RNase) activity. Hirs *et al.* (2) demonstrated that acid extracts of pancrease prepared according to Kunitz (3) contained these components, named RNase A and RNase B, in the approximate proportions of 10:1. Chemically, RNase A and B have been reported to differ by a single carboxyl group (4). Recently, Plummer and Hirs (5) reported that RNase prepared from bovine pancreatic juice possesses an amino acid composition identical to that of RNase A. They found that RNase B contained five residues of mannose and two residues of glucosamine per mole of protein not contained in RNase A. These authors suggested that crystalline pancreatic RNase may contain RNase A, RNase B, and modified forms of RNase B derived by partial removal of carbohydrate.

The antigenic specificities of RNase A and B are not distinguishable by serological methods (6, 7). The two components can be separated by electrophoresis in agar, but the immune reaction in gel, during the second phase of the immunoelectrophoretic technique, results in a continuous precipitin band between the two components (6). Hence, the chemical group responsible for the difference in mobilities between RNase A and B does not participate in the antigenic determinant of the protein.

Bingham and Zittle (8) recently isolated two ribonucleases from bovine milk and compared them with the pancreatic enzymes. Milk RNase A, the major component, appeared to be identical with pancreatic RNase A by electrophoresis, specific activity, and amino acid composition. On cellulose acetate electrophoresis, the milk RNase B showed two components, one of which appeared to be RNase A. This report presents evidence of serological identity between pancreatic RNase and RNase from milk.

EXPERIMENTAL[2]

Ribonuclease Preparations

The RNase preparations were furnished by C. A. Zittle and E. W. Bingham, Eastern Regional Research Laboraotry, U. S. Department of Agriculture. S-RNase was a pancreatic preparation from Sigma Chemical Company (Lot R71B-202). Z-RNase was prepared by Zittle by crystallizing twice from $(NH_4)_2SO_4$ by the method of Kunitz (3). These two pancreatic preparations had the same enzymic activity as determined by the method of Eichel *et al.* (9). The milk RNase prep-

[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[2] Mention of products in this paper does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

arations were purified by the methods described (8) but were not chromatographed. M80-RNase had 80% and M50-RNase had 50% of the enzymic activity of S-RNase.

Bovine Serum

Two samples of bovine serum, one from a lactating cow and one from a young bull, were supplied by Dr. M. L. Crandall, Animal Husbandry Research Division, U. S. Department of Agriculture. The sera were separated from the freshly clotted blood, immediately frozen, transported to the laboratory, and stored at −20°C.

Production of Rabbit Antisera

Four rabbits weighing about 2.5 kg were immunized to the pancreatic ribonucleases by two weekly subcutaneous injections of antigen (2 mg protein per dose) in Freund's complete adjuvant (10). After an interval of 3 weeks, each animal was inoculated intravenously with a series of 4 daily doses of 2 mg of antigen in saline solution. The rabbits were bled from the heart 5 days after the final injection. Two animals were immunized with Z-RNase, antisera 214 and 215, and two with S-RNase, antisera 216 and 217.

Ouchterlony Analyses

Ouchterlony (11) plates were prepared by the methods devised by Feinberg (12, 13). Flat-bottom Petri dishes,[3] 6 cm internal diameter, were coated with silicone to prevent underrunning of the reactants. Eight ml of 0.5% molten Ionagar No. 2[3] were poured into the Petri dishes. After the agar had set, the desired gel patterns were formed with Feinberg agar cutters.[3] Antiserum was added to the serum well and usually allowed to diffuse several hours. The antigen wells were then filled and the diffusion patterns were developed in moist atmosphere at 24° ± 1° for 2-4 days.

RESULTS

The relative precipitating potencies of the RNase antisera and the optimal concentration of antigen for Ouchterlony tests were determined as illustrated in Fig. 1. The six inner wells were filled with Z-RNase antiserum 215. Four hours later the outer wells were filled with dilutions of S-RNase beginning with a concentration of 1.024 mg per milliliter in well 1 and proceeding clockwise with 2-fold serial dilutions of the antigen. Figure 1 shows the diffusion pattern after 42 hours. Two main precipitin zones were observed: a strong inner zone that extended to well 10, which contained 0.002 mg S-RNase per milliliter, and an outer band that extended to well 6, which contained 0.032 mg S-RNase per milliliter. The inner zone appeared to be partially resolved into two bands in the higher dilutions of antigen.

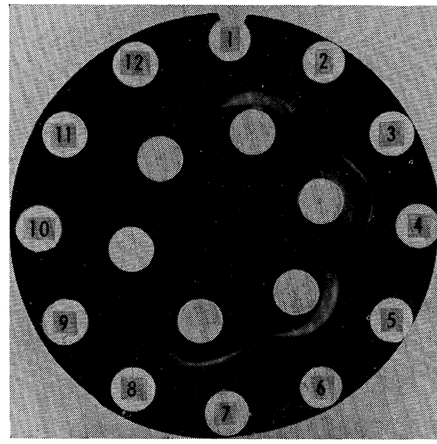

Fig. 1. Diffusion pattern of S-RNase (outer wells) with Z-RNase antiserum (unlabeled inner wells).

Essentially the same results were obtained when S-RNase diffused against S-RNase antisera 216 and 217, and against Z-RNase antiserum 214.

When Z-RNase diffused under similar conditions against either S-RNase or Z-RNase antisera, the inner zone showed the same characteristic as that shown in Fig. 1, but the outer band was not visible beyond wells 2 or 3.

When M80-RNase, from milk, diffused against the pancreatic RNase antisera, only one precipitin zone appeared on the plates. This band showed the same characteristics as the inner band of the pancreatic preparations except that precipitation was not visible beyond well 9, which contained 0.004 mg of M80-RNase per milliliter.

From the above results it was concluded that the outer precipitin bands represented a contaminant in both preparations of pancreatic ribonucleases and that S-RNase contained more of the contaminant than Z-RNase. The trace of contaminant in Z-RNase was sufficient to induce formation

[3] Purchased from Consolidated Laboratories, Inc., Chicago Heights, Illinois.

of antibody in rabbits but was not sufficient to form visible precipitates in any of the antisera except when used in relatively high concentration. The absence of the outer band in the diffusion pattern of milk RNase indicated that the contaminant originating from the pancreas was not present in the milk preparations.

Figure 2 shows the diffusion pattern obtained with the four RNase preparations and S-RNase antiserum 217. The central antiserum well was filled and allowed to diffuse 24 hours. The antigen wells were then filled with RNase solutions containing 0.15 mg of protein per milliliter. The principal precipitin bands from each preparation merged with neighboring bands, which indicated identical specificity. The outer bands originating from wells containing S-RNase were undoubtedly due to the same contaminant observed in Fig. 1. This contaminant in Z-RNase was also demonstrated in some of the plates by a barely discernable outer band which merged with the corresponding band from S-RNase.

Patterns similar to Fig. 2 were observed in a series of four Ouchterlony plates in which the antigen concentration ranged, in 2-fold dilutions, from 0.128 to 0.016 mg per milliliter. The outer band from S-RNase was not visible at the lowest concentration. Moreover, at the lower concentrations, the hexagonal pattern tended to elongate toward the well containing M50-RNase due to the lower concentration of specific antigen in that preparation.

Figure 3 records an attempt to resolve the precipitin bands of ribonucleases derived from the pancreas and from milk by using a gel pattern designed for this purpose (13). The eccentrically placed inner well was filled with S-RNase antiserum. The first four antigen wells were filled with M80-RNase, and the second four wells were filled with Z-RNase. Both solutions contained 0.128 mg of antigen per milliliter. The results, recorded after 4 days, showed a dense outer band and a faint inner band. Corresponding bands from each preparation joined between wells 4 and 5. The significance of the inner band was not further investigated.

Figure 4 shows that the presence of RNase in cow's milk can be detected directly by the Ouchterlony technique. In this test the center well was filled with S-RNase antiserum. Nineteen hours later the antigen wells were filled with: skim milk, Z-RNase (0.064 mg per milliliter), M80-RNase (0.064 mg per milliliter), cow serum, bull serum, and with a skim milk preparation in which the calcium ion has been replaced with sodium (Milk Na). The results recorded in Fig. 4, after diffusion for 48 hours, showed clearly that a precipitin band originating from skim milk merged with the band from Z-RNase which in turn merged with the band from M80-RNase. No precipitin band was visible from either of the bovine sera. In control tests, skim milk gave no precipitate with normal rabbit serum; pretreatment of

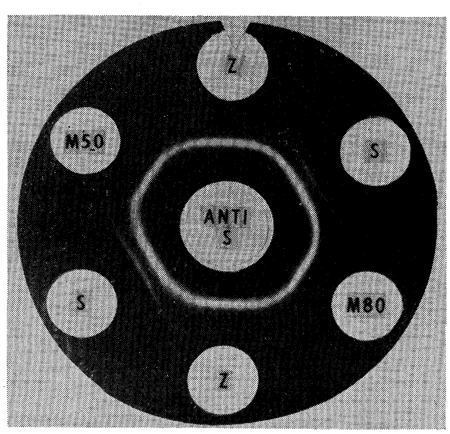

FIG. 2. Comparison of milk RNase and pancreatic RNase.

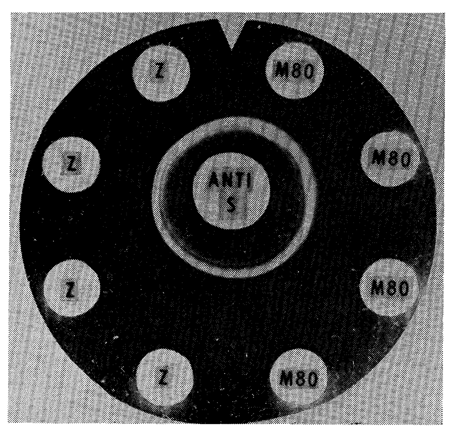

FIG. 3. Attempt to resolve precipitin bands of M80-RNase and Z-RNase.

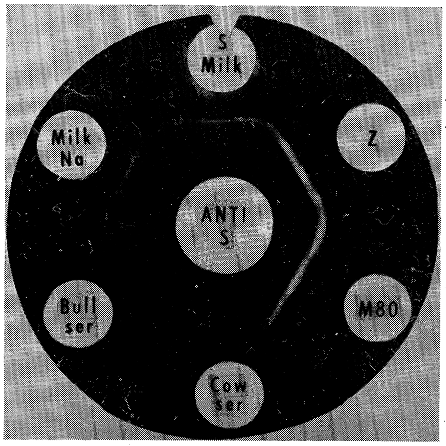

FIG. 4. Demonstration of RNase in skim milk. Ribonuclease was not detected in cow serum or bull serum.

skim milk with RNase antiserum inhibited formation of the precipitin band.

In other experiments a crude extract of beef heart produced a faint band when diffused against S-RNase antiserum. This band crossed both the inner and outer bands produced by S-RNase with S-RNase antiserum. The heart extract did not produce a band when diffused against Z-RNase antiserum. Hence, S-RNase apparently contains at least two contaminants.

DISCUSSION

Some enzymes have been reported to exist in more than one structural form within the same animal body. For example, serological techniques have distinguished differences between muscle and liver phosphorylases (14). Lactic dehyrogenase isolated from adult chicken heart muscle differs completely from the lactic dehydrogenase found in breast muscle as judged by physical, enzymic, and serological criteria (15). Ribonuclease isolated from different animal species shows distinguishable serological properties but exhibits similar specific enzymic activity (16). It was pertinent, therefore, to determine whether RNase isolated from milk was serologically identical to pancreatic RNase.

The gel-diffusion studies reported here clearly demonstrate the serological identity of milk and pancreatic ribonucleases and confirm the conclusions of Bingham and Zittle (8) based on their physical and enzymic characterization of RNase from the two sources. As might be expected, the milk preparations were less potent in antibody precipitating activity than the pancreatic RNase because neither of the milk ribonucleases was purified to the same extent as the pancreatic preparations. For this reason and because of the limited supply of material available, quantitative precipitin studies were not conducted. Accordingly, quantitative statements of the serological relationship of milk RNase to pancreatic RNase cannot be made at this time.

Other workers (6) have observed the presence of an enzymically inactive contaminant that was antigenically distinct from RNase in crystalline preparations of the enzyme. In the present study two distinct contaminants were detected in S-RNase. One of these was readily demonstrated in Ouchterlony tests when S-RNase was diffused against S-RNase antiserum. Antibodies to the second contaminant were revealed when a crude extract of beef heart was diffused against S-RNase antiserum. The first contaminant was also detected, in lesser concentration, in Z-RNase. The second contaminant was not detectable in Z-RNase, nor were specific antibodies for it present in Z-RNase antiserum.

Taborsky (17) suggested that the contaminant in crystalline RNase preparations may be nucleotide material. Nucleotides are haptenic (18), but their capacity to induce antibody formation is questionable.

Undoubtedly contaminants were present also in the milk RNase preparations, but they did not react with antibodies to contaminants in the S-RNase antisera. Pancreatic RNase antisera might therefore be useful as a homogeneous antibody system for studying milk RNase preparations.

The failure to detect RNase in bovine serum was probably a matter of sensitivity of the serological method used. Connolly *et al.* (19) reported that whole human blood contains an RNase concentration equivalent to that of 0.1 μg per milliliter of crystalline pancreatic RNase. Other workers (20) have reported as much as 0.6 μg per milliliter in human serum (cf. 19, 21). These levels of RNase are below the level of sensitivity of the serological method used in these studies.

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